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## The Human Longevity Gene Homolog *INDY* and Interleukin-6 Interact in Hepatic Lipid Metabolism

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## Abstract

Reduced expression of the *Indy* (*I am Not Dead, Yet*) gene in lower organisms promotes longevity in a manner akin to caloric restriction. Deletion of the mammalian homolog of *Indy* (*mIndy*, *Slc13a5*) encoding for a plasma membrane associated citrate transporter expressed highly in the liver, protects mice from high-fat diet and aging-induced obesity and hepatic fat accumulation through a mechanism resembling caloric restriction. We aimed to study a possible role of *mIndy* in human hepatic fat metabolism. In obese, insulin resistant patients with NAFLD, hepatic *mIndy* expression was increased and *mIndy* expression was also independently associated with hepatic steatosis. In non-human primates, a two year high fat, high sucrose diet increased hepatic *mIndy* expression. Liver microarray analysis showed that high *mIndy* expression was associated with pathways involved in hepatic lipid metabolism and immunological processes. Interleukin-6 (IL-6) was identified as a regulator of *mIndy* by binding to its cognate receptor. Studies in human primary hepatocytes confirmed that IL-6 markedly induced *mIndy* transcription via the IL-6-receptor (IL-6R) and activation of the transcription factor Stat3 and a putative start site of the human *mIndy* promoter was determined. Activation of the IL-6-Stat3 pathway stimulated *mIndy* expression, enhanced cytoplasmic citrate influx and augmented hepatic lipogenesis *in vivo*. In contrast, deletion of *mIndy* completely prevented the stimulating effect of IL-6 on citrate uptake and reduced hepatic lipogenesis. These data show that *mIndy* is increased in liver of obese humans and non-human primates with NAFLD. Moreover, our data identify *mIndy* as a target gene of IL-6 and determine novel functions of IL-6 via mINDY. Targeting human mINDY may have therapeutic potential in obese patients with NAFLD.

## Keywords

Indy; Liver; NAFLD; Insulin Resistance; IL-6

## Introduction

Reduced *Indy* (*I am Not Dead, Yet*) gene expression in *D. melanogaster* and *C. elegans* promotes longevity in a manner akin to caloric restriction in most studies (1–4). Long-lived flies with lower *Indy* expression have decreased whole body fat stores, lower expression of insulin-like proteins, and increased mitochondrial number (3, 5). The *Indy* gene product is a cation-independent, electroneutral tricarboxylate carrier (6, 7), able to transport citrate across the plasma membrane as its preferred substrate. *Indy* is highly expressed in organs

involved in energy homeostasis in flies (8). In mammals, the gene product of *Slc13a5* (*mIndy*) encodes the sodium-coupled citrate transporter NaCT (mINDY); it shares the highest sequence and functional similarity with *D. melanogaster* INDY (9) and is highly expressed in liver tissue (6, 7, 10–12). In mammals, mINDY mediates an electrogenic cotransport of a various di- and tricarboxylates, with the highest affinity for citrate (6). Citrate and succinate are mINDY substrates with the highest plasma concentrations (11). The amino acid sequence of the N-terminal sodium- and the carboxy-binding pocket are highly conserved between many species, from bacteria to mice to human (13).

Recently, our lab translated findings from *Drosophila* into mammals by showing that *mIndy* deletion (*mINDY*<sup>-/-</sup>) mimics important aspects of caloric restriction in mice (7), without reducing caloric intake. *mINDY*<sup>-/-</sup> mice gain less weight on a high-fat diet and during the aging process together with lower liver fat content and reduced insulin resistance (7). Furthermore, we showed that liver specific *mIndy* knockdown using anti-sense oligonucleotides (ASOs) in adult rats reduced hepatic lipid storage and enhanced hepatic insulin sensitivity upon feeding a HFD (14). Finally, a competitive, stereo sensitive small molecule inhibitor of the mINDY transporter offered complete protection from diet-induced glucose intolerance in mice and ameliorated diet-induced fatty liver disease as shown by an independent research group (15).

The cytokine interleukin-6 (IL-6) is a pleiotropic cytokine with a complex role in inflammation and metabolic disease. IL-6 binding to its cell surface receptor with subsequent activation of janus family kinases and phosphorylation of signal transducer and activator of transcription 3 (STAT3), promoting its nuclear translocation, DNA binding, and subsequent target gene expression, including important genes of the acute phase reaction. Moreover, PI-3 phosphorylation through IL-6 results in Akt activation. IL-6 also activates the pro-oncogenic Ras/Raf/MAPK 1/2 signaling pathway. In obesity and the metabolic syndrome, cytokines, such as IL-6, are highly secreted from adipose tissue and have been proposed to promote accompanying metabolic diseases, i.e. insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) (16–19). However, anti-inflammatory and beneficial metabolic actions of IL-6 have been reported as well (20–22). Thus, the exact role of IL-6 in metabolic regulation remains highly controversial (23).

Given the therapeutic potential of mINDY inhibition in metabolic disease and aging, our aim was to investigate the role of the human *mIndy* homolog in the pathophysiology of obesity and NAFLD, and the regulation of its expression under conditions associated with obesity. We observed that *mIndy* expression was increased in obese patients with NAFLD and that the cytokine IL-6 is a potent regulator of the human *mIndy* homolog.

## RESULTS

### Human *mIndy* Tissue Distribution

To determine *mIndy* distribution in human subjects, semi-quantitative PCR measurements of *mIndy* expression were performed in different tissues. *mIndy* expression was markedly higher in liver tissue compared to all other tissues studied (Figure 1A), with skeletal muscle

and colon showing minimal *mIndy* expression levels confirming previous reports in mice (7), rats (12, 14) and human subjects (11, 24).

### Transport kinetics of human mINDY

Human *mIndy* (*SLC13A5*) cDNA was cloned, transfected into HEK293 cells, and the uptake capacities of mINDY for citrate and succinate were analyzed. mINDY proved to be a high capacity transporter for citrate over a broad range of concentrations (0–5000  $\mu\text{M}$ ), which encompasses physiological concentrations (25) ( $K_m=2254\pm 207\ \mu\text{M}$ ,  $V_{\max}=25117\pm 1051\ [\text{pmol}/\text{mg}\cdot\text{min}^{-1}]$ ) and a succinate transporter with an intermediate capacity ( $K_m=5156\pm 665\ \mu\text{M}$ ,  $V_{\max}=1046\pm 71\ [\text{pmol}/\text{mg}\cdot\text{min}^{-1}]$ ) (Figure 1B and 1C).

### Human hepatic *mINDY* mRNA expression is increased in insulin resistant patients with obesity and nonalcoholic fatty liver disease

Forty-nine liver samples were collected from patients undergoing hepatic surgery and processed for the quantitative determination of *mIndy* mRNA levels. With respect to current diagnostic criteria (26) all subjects presenting with  $\geq 5\%$  steatotic hepatocytes in our histopathological analysis were considered as having NAFLD. Accordingly, 37% of our study subjects had no sign of any pathological condition in the respective liver tissue, compared to 63% suffering from NAFLD. Liver samples were collected before the blood supply to the liver was clamped (Pringle maneuver) to minimize cold ischemia. Characteristics of patients are given in Table 1. *mIndy* expression was positively correlated to BMI ( $r=0.361$ ,  $P=0.011$ , Figure 2A), waist circumference ( $r=0.418$ ,  $P=0.003$ , Figure 2B), body fat ( $r=0.441$ ,  $P=0.020$ , Figure 2C), and nominally for insulin resistance as assessed by HOMA-IR ( $r=0.333$ ,  $P=0.019$ ), and hepatic insulin resistance index ( $r=0.316$ ,  $P=0.027$ ). Of note, the strongest correlation was present with histology determined steatosis ( $r=0.688$ ,  $P<0.001$ , Figure 2D). To determine whether *mIndy* expression was independently associated with hepatic steatosis, we adjusted for several potential confounders, including age, sex, waist circumference and insulin resistance, by means of multivariate linear regression analysis. The degree of liver histology-determined steatosis remained significantly associated with *mIndy* expression (Model in Table 1B), suggesting that *mIndy* independently predicts human hepatic steatosis. Total RNA microarrays from liver samples of patients with low ( $1\pm 0.1$  AU) and high *mINDY* expression ( $3.4\pm 0.4$  AU), matched for BMI and other metabolic parameters, except for hepatic fat content ( $34\pm 5\%$  versus  $16\pm 2\%$ , for high versus low expressed *mIndy*, respectively,  $P=0.02$ ,  $n=3$ ) revealed biological processes involved in hepatic lipid metabolism and the acute phase response being activated in patients with high *mIndy* expression in gene ontology enrichment analysis (Table 2).

### High fat, high sucrose diet increases hepatic *mIndy* expression in non-human primates

To independently confirm our human data, we used a cohort of non-human primates raised in a controlled environment. Fourteen middle-aged male rhesus monkeys were randomized into two groups and fed either a standard diet (SD,  $n=4$ ) or a high-fat, high sucrose (HFS) diet ( $n=10$ ) for two years, as described (27). Monkeys fed a HFS diet showed significant increases in body weight (Figure 3A), waist circumference (Figure 3B) and serum IL-6 levels (Figure 3C) when compared to SD-fed animals. Total RNA microarrays from monkey liver samples, and pairwise comparison between HFS and SD cohorts indicated a 3.5-fold

increase in the z-ratio for *mIndy* in response to HFS. These data were confirmed by RT-PCR, with a  $3.7 \pm 0.7$  fold increase in hepatic *mIndy* expression in obese, HFS-fed monkeys as compared to SD-fed controls (Figure 3D). These data show that a high fat high sucrose diet or the resulting diet induced obesity increases *mIndy* expression in non human primates.

Similarly, in non-fasted male 16 week high fat diet fed C57BL/6 mice, hepatic *mIndy* expression was induced  $2.5 \pm 1.0$  fold in comparison to the SD-fed group (SD,  $n=4-5$ ;  $1.0 \pm 0.2$ ) (Figure 3E). Interestingly, in a murine NASH model (high fat/methionine low-choline deficient diet=HFD-CD), hepatic *mIndy* expression was markedly increased in HFD-CD-fed mice ( $n=4$ ) with  $> 20$ -fold increase (Figure 3F) after 6 weeks of the intervention. The increase of *mIndy* expression in the course of NASH was also time-dependent (data not shown). Since these data suggest that *mIndy* is regulated by an inflammatory mediator, expression levels of *mIndy* in murine hepatocytes and Kupffer cells were analysed independently. In Kupffer cells, *mIndy* expression was very low, on a normal diet as well as on a HFD (Figure S1A).

### The cytokine interleukin-6 (IL-6) is positively associated with hepatic *mIndy* expression

Other inflammatory mediators might also induce *mIndy*. Total RNA microarrays suggested that high *mIndy* expression was associated with activation of some acute phase response components (Table 2). The acute phase response, which serves as a core of the innate immune system, is initiated and mediated by a number of cytokines, most importantly by IL-6 (28). IL-6 independently predicted the degree of hepatic steatosis at a cut off level of 4.81pg/ml (29, 30). Therefore, patients were grouped according to serum IL-6 levels below or above this threshold. In patients with plasma IL-6 levels above this threshold, *mIndy* expression was twofold higher compared to patients with IL-6 levels below this threshold ( $P=0.008$ ) (Figure 4A). In a cross sectional comparative analysis of serum from these patients, hepatic *mIndy* expression positively correlation with circulating IL-6 levels after adjustment for liver fat ( $r^2=0.300$ ;  $P=0.038$ , data not shown).

### IL-6 induces hepatic *mIndy* expression in primary human hepatocytes and in mice

Treatment of human primary hepatocytes with IL-6 (50ng/ml) induced *mIndy* expression in a time-dependent manner, with a  $42 \pm 14$  fold increase after 24 h of IL-6 ( $P=0.031$ ) (Figure 4B). Moreover, blockade of IL-6 signalling with the human monoclonal IL-6 receptor antibody tocilizumab completely abolished IL-6's ability to increase *mIndy* expression (Figure 4C).

To assess the ability of IL-6 to recapitulate this effect *in vivo*, IL-6 was injected intravenously into C57BL/6 wildtype mice. In this setting, hepatic *mIndy* expression was induced by  $3.5 \pm 0.3$  fold after 240 min (Figure 4D). To independently verify the role of the cognate IL-6 receptor in the regulation of *mIndy* expression, liver samples of mice with a targeted liver-specific deletion of the IL-6 receptor (IL-6R<sup>L-KO</sup>) were analyzed, which were generated by crossing IL-6R fl/fl mice to ALFP-Cre recombinase mice as described in (21). Deleting the hepatic IL-6 receptor led to a marked  $68 \pm 4\%$  reduction in hepatic *mIndy* expression when compared to IL-6Rfl/fl mice (Figure 4E). Total RNA microarrays from these liver samples indicated that  $\sim 900$  genes were reduced in IL-6R<sup>L-KO</sup> mice compared to



IL-6R fl/fl. *mIndy* ranked 20<sup>th</sup> among the top negatively regulated genes. Together, these data confirm that IL-6 regulates *mIndy* transcription in both human and mouse livers via the IL-6 receptor.

To determine whether the action of IL-6 can also be attributed to an endocrine-mediated mechanism, human non-parenchymal cells, including Kupffer cells, were co-cultivated with hepatocytes, and then stimulated with lipopolysaccharide (LPS), which provides a strong stimulus for macrophages to secrete an array of pro-inflammatory cytokines, including IL-6. In this setting, *mIndy* expression was induced 5-fold (Figure 4F), and pre-incubation of the co-culture with tocilizumab largely reduced the induction of *mIndy* by LPS stimulated cells. When hepatocytes were cultured without non-parenchymal cells, such as Kupffer cells, the LPS response was reduced but not completely prevented (Figure S1B). Together, these data suggest that IL-6, but not other cytokines, induces *mIndy* in an inflammatory paracrine and endocrine-related setting, because IL-6 receptor blockade largely abolished the response.

### Human *mINDY* is induced via the transcription factor Stat3

5'-RACE experiments were performed to identify the transcription start site of the human *mIndy* (*SLC13A5*) gene, with gene-specific primers for reverse transcription in exon 6 and nested PCR primers located in the fifth and third exons. A possible transcription start site located approximately 40 bp upstream of the 5'-end of the published start ATG was found in 5 out of 7 clones that contained sequences continuing into the first exon (Figure 5A). RT-PCR was used to verify the putative transcription start site. Genomic DNA and cDNA were amplified with specific primers, whereby the forward primers were either located 26 bp upstream of the potential transcription start site or at the published ATG start codon. Normalized *mINDY* template numbers did not differ between cDNA and genomic DNA when using the primer directed at the ATG start codon, whereas the normalized *mIndy* template number in the cDNA was reduced about 95% versus genomic DNA using the primer upstream of the putative transcription start site. Hence, we tentatively assigned the 5'-upstream region of the latter transcription start site as promoter region. *In silico* analysis of the 618 bp human *mIndy* promoter fragment revealed a potential Stat responsive element sequence as shown by MatInspector software. Moreover, a second Stat responsive element was identified with the Stat consensus sequence TT(N<sub>4-6</sub>)AA (Figure 5A, 5C). Two reporter gene constructs were generated by cloning either a stretch of 618 bp containing both Stat responsive elements or a fragment of 376 bp length including one Stat binding motif upstream of the start ATG of the human *mIndy* gene, including the putative transcription start site, in front of luciferase in the vector pGL3-basic. Transiently transfection of mouse hepatocytes with these Stat-containing human *mINDY* promoter constructs increased the luciferase activity about 53 % and 138 %, when either one, or both Stat responsive elements were present, respectively (Figure 5B). To determine which of the elements is indispensable for the induction of *mINDY* by IL-6, mutations were introduced into both Stat3-binding elements at the 376 bp and 618 bp *mIndy* promoter fragments by PCR-based site-directed mutagenesis (Figure 5C). Surprisingly, none of the mutations in the potential Stat3-binding sites reduced *mINDY* promoter activity when stimulated with IL-6 (Figure 5D). These data suggest that other binding sites, an indirect mechanism or non-transcriptional effects

contribute to the Stat3-dependent transactivation of the *mIndy* gene following IL-6 stimulation.

### IL-6 mediated metabolic effects via *mINDY*

mINDY is a transporter of citrate from the interstitial space into the cytosol. Incubation of primary human hepatocytes with IL-6 (50ng/ml) in the presence of physiological concentrations of citrate (10μmol/l) led to a significant increase both in <sup>14</sup>C- citrate uptake (Figure 6A) and lipogenesis from citrate (Figure 6B). Deletion of *mIndy* in primary hepatocytes isolated from *mINDY*<sup>-/-</sup> mice (7) completely prevented these effects of IL-6 (Figure 6C and 6D). To determine whether this also has a role in the in vivo setting, we generated hepatocyte specific mINDY KO mice as described in the methods section. In these mice, mINDY expression was completely abolished in the liver, but not other tissues such as adipose tissue, muscle and brain (data not shown) compared to mINDY fl/fl mice. IL-6 was infused for 14 days using mini osmotic pumps as described in (31) into mINDY-L-KO and mINDY fl/fl. mINDY fl/fl mice without IL-6 infusion were used as negative control. One week prior to the experiment, an intravenous line was inserted into the right jugular vein. On the day of the experiment, mice were fasted overnight and infused with 7μCi <sup>14</sup>C-Citrate continuously and livers were taken after 4 hours of infusion. Fatty acid synthesis from citrate was determined. Our data show that chronic infusion of IL-6 for 14 days nearly doubled fatty acid synthesis from citrate in livers of mINDY fl/fl mice. Liver specific deletion of *mIndy* completely prevented IL-6 induced fatty acid synthesis from citrate. These data suggest that mINDY mediates IL-6 induced hepatic lipid synthesis from citrate in mice in vivo (Figure 6E). In line with these findings, HEK293 cell lines with stable 50 fold overexpression of *mIndy* lead to a concentration dependent uptake of citrate (Figure 1B). Citrate induced lipogenesis increased by more than 130 fold in these cells (Figure S2). Taken together, these results suggest a novel function of IL-6, namely to stimulate hepatocyte citrate uptake and hepatic citrate induced lipogenesis *in vivo*. The effect of IL-6 is mediated via *mINDY*.

### Significance of human mINDY compared to mouse and rat mINDY

Finally, a recent publication raised questions about the translatability of mINDY as a target for the treatment of metabolic disease from the mouse to the human situation due to differences in uptake properties between species (32). Therefore, we directly measured <sup>14</sup>C- citrate uptake into mouse, rat, and human primary hepatocytes incubated with low physiological concentrations of citrate. In this setting, citrate uptake was highest in human primary hepatocytes, followed by mouse and rats (Figure 6F). These data directly show that human mINDY takes up citrate even in low physiological doses and are rather supportive for the notion that human mINDY is a viable target for the treatment of metabolic disease.

## DISCUSSION

Reduced *Indy* gene expression increases life span in lower organisms in most (1, 3, 7, 33) but not in all (34) studies. Moreover, deletion or knock down of *Indy* or its homologs lead to a lean phenotype by mechanisms akin to caloric restriction, without a reduction in food intake (1, 3, 7, 33). Here, we provide new data showing similar associations in humans. In



lean subjects with low amounts of liver fat, *mIndy* expression was low, and *mIndy* expression increased with increasing BMI and liver fat content. Furthermore, our data from non-human primates indicate that *mIndy* is regulated in response to a ‘unhealthy’ high fat, high sucrose diet. In mice, a high fat diet but to a larger extent a high fat- NASH inducing diet (methionine low, choline deficient), also induced *mIndy* in the liver. Taken together, these data show for the first time that *mIndy* is regulated by the nutritional state and might therefore play a pathophysiological role in diet induced obesity and associated conditions, such as NAFLD.

Our data suggest that the association between *mINDY*, obesity and NAFLD was mediated, at least in part, by the cytokine IL-6. Moreover, we provide evidence for human *mIndy* being a target gene of Stat3 in response to IL-6. Both, the treatment of primary human hepatocytes with IL-6 and intravenous administration of IL-6 in mice markedly increased hepatic *mIndy* mRNA levels. This gene encodes the plasma membrane citrate transporter mINDY (NaCT, SLC13A5) and, thus, stimulation of human and rodent hepatocytes with IL-6 enhanced cellular uptake of citrate. Citrate, in turn, is a precursor of acetyl-CoA and fatty acid in the liver. *mIndy* induction in mice *in vivo* and in murine and human primary hepatocytes *in vitro* required the cognate IL-6 receptor. Indeed, the monoclonal human IL-6R antibody tocilizumab abolished the effect of IL-6 on *mIndy* expression and IL-6R<sup>L-KO</sup> mice displayed significantly lower hepatic *mIndy* levels. Our data show that two Stat responsive elements by *in silico* analysis of the human *mIndy* promoter 5’ upstream of the putative transcription start site, consistent with the notion that Stat3 transcriptional activity induces the human *mIndy* promoter. Indeed, reporter gene constructs containing the two Stat binding sites were activated by IL-6. However, mutating these Stat-binding sites did not result in reduced *mIndy* promoter activity. These data suggest that either i) other enhancer-elements, far more upstream of the transcription site mediate the effect, ii) the induction is mediated indirectly via Stat3-dependent induction of other transcription factors, iii) *mIndy* mRNA is regulated at the posttranscriptional level. More studies are needed to clarify these possibilities.

After stimulation of human non-parenchymal cells, including Kupffer cells, with LPS, inducing an array of cytokines, IL-6 receptor blockade attenuated *mIndy* induction in hepatocytes. When only hepatocytes were cultured, the LPS response was reduced. These data suggests that IL-6 likely is a cytokine contributing to the induction of *mIndy* in a pro-inflammatory setting and that the effect is mediated in both, a paracrine and endocrine manner from immune competent cells. Collectively, these data provide evidence for IL-6 to induce the human longevity gene homolog *mIndy* via Stat3 in human and rodent liver tissues.

IL-6 is increased in obesity, T2D and NAFLD. Obesity and NAFLD are characterized by a chronic inflammatory state (35). The nature of the immune response is unique as compared to an acute inflammatory responses and has a well characterized impact on metabolic regulation (35). In obesity, accumulation of immune cells such as macrophages and T-lymphocytes in the liver and white adipose tissue leads to increased secretion of IL-6 (35). The effect of IL-6 on hepatic lipid metabolism has been investigated with diverging results (20–22, 31, 36–40). IL-6 transgenic animals were protected from HFD induced obesity and fatty liver. IL-6 levels in these animals are, however, several fold higher than IL-6

concentrations in obesity (39). In studies in IL-6-deficient, and IL-6R deficient mice, the course of diet-induced hepatic steatosis support a protective effect of the cytokine in obesity and fatty liver disease (41, 42). In these animals, a compensatory increase in many other cytokines has been reported (21). In contrast, low dose application of murine IL-6 in mice lead to hepatic steatosis (43). Subchronic IL-6 administration in IL-6-deficient mice exacerbated hepatic steatosis by increasing lipogenesis (44). Importantly, neutralization of IL-6 with neutralizing antibodies in HFD fed mice improved glucose tolerance, ameliorated liver fat content (45). Longterm IL-6 incubation of rat hepatocytes increased lipid synthesis (Brass and Vetter et al., 1994), i.p. IL-6 administration in mice stimulated hepatic lipogenesis by increasing hepatic citrate (46) and acetyl-CoA concentrations (19). Taken together, these data show that IL-6 is a pleiotropic cytokine with complex roles in metabolic disease. Our data from patients and non-human primates confirm the association between IL-6 and anthropometric measures of obesity and hepatic lipid content. Moreover, our data show an association between *mIndy* expression and IL-6 as well as obesity and NAFLD. Our *in vitro* studies corroborate the observation by showing that IL-6 induced *mIndy* expression in primary hepatocytes from human and mice, and that IL-6 increased hepatic citrate uptake and hepatic lipogenesis from circulating citrate via mINDY *in vivo*.

Circulating citrate concentrations range from 50–150  $\mu\text{mol/L}$ . We provide evidence that human mINDY is a high capacity transporter for circulating citrate, which is hardly saturated by circulating concentrations. Thus, mINDY allows a continuous uptake of citrate into hepatocytes. Citrate is a central metabolite, both, in cytosolic and in mitochondrial metabolism, by connecting carbohydrate catabolism and lipogenesis. Citrate is the main carbon source of fatty acid synthesis which has recently been shown to be enhanced by IL-6 (19). Moreover, citrate acts as an allosteric activator of acetyl-CoA carboxylase (ACC). Cytosolic citrate also contributes to NADPH generation via malic enzyme for lipogenesis (47). In line with this mechanism, siRNA mediated *mIndy* knockdown reduced total fat content in human hepatocytes (24). Knockdown of *mIndy* in rats using ASOs (14) and in mice using siRNA (48) reduced liver fat content when rats were fed a HFD. Most importantly, inhibition of mINDY using a novel small molecule improved glucose tolerance and ameliorated liver fat content upon high fat feeding in mice (15). Conversely, our data show that overexpression of mINDY in HEK293 cells resulted in an increase in citrate uptake and intracellular fatty acid and sterol synthesis. Moreover, enhancing the activity of citrate transport by mINDY in HepG2 cells augments citrate-induced lipid synthesis (12, 49). Inducing *mIndy* in obesity and NAFLD, might, thus, contribute to the pathogenic process by enhancing the uptake of citrate which is then used to fuel hepatic lipid synthesis.

In summary, our data show that the longevity gene homolog *mIndy* was regulated in humans according to the nutritional state and that IL-6 increased human hepatocyte *mIndy* expression in a paracrine and endocrine manner. Our work describes a promoter sequence of human *mIndy* that is located upstream of the most frequent transcription start site, which was determined by 5'-RACE. We also describe a novel function of IL-6, namely the induction of citrate uptake into human primary hepatocytes via mINDY and by this mechanism, an induction of lipid synthesis in human primary hepatocytes. Based on our data in humans, future studies are needed to address the important question whether or not the

inhibition of human mINDY is beneficial in the treatment of metabolic disease, such as obesity, NAFLD and type 2 diabetes.

## EXPERIMENTAL PROCEDURES

### Patients

*mINDY* gene expression in different tissues was measured in the Human Total RNA Panel (BD Biosciences Clontech, Heidelberg, Germany), supplemented with RNA from human liver from our laboratory. For liver samples and hepatocytes, experimental procedures were performed according to the guidelines of the charitable state-controlled foundation Human Tissue and Cell Research, with the informed patient's consent approved by the local Ethical Committee of the Charité University School of Medicine Berlin (EA2/135/08). All subjects gave written informed consent at least 24 hours prior to surgery. Forty-nine patients were enrolled between February 2009 and March 2010. Details regarding inclusion and exclusion criteria have been published (50), German Clinical Trials Register: DRKS00005450.

### Preparation and cultivation of primary human hepatocytes

Tissue samples from liver resections were obtained from patients undergoing partial hepatectomy. The experimental procedures were performed according to the guidelines of the charitable state-controlled foundation Human Tissue and Cell Research, with the informed patient's consent approved by the local Ethical Committee of the Charité University School of Medicine Berlin (EA2/007/13). Detailed information is given in the Supplemental Section.

### Animals

**Non-human primates**—Studies were performed as described (27). Detailed information is given in the Supplemental Section.

**Mice**—Generation of mINDY-KO mice and IL-6R $\alpha$ <sup>L-KO</sup> mice have been described previously (7, 21). Detailed information is given in the Supplemental Section.

Generation of liver specific mIndy KO mice: We previously generated a targeting vector, in which exons 1 to 6 of the SLC13A5 gene were flanked by loxP sites (7). After yielding SLC13A5 (mIndy) fl/fl, these mice were crossed with Albumin-Cre mice {Kellendonk 2000}, and heterozygous animals were further intercrossed to mIndy<sup>FL/FL</sup> Alb-Cre, i.e., hepatocyte-specific mIndy knockout mice on a C57/BL6 background (mIndy L-KO).

Male C57BL/6J mice were purchased from Charles River Laboratories. First mice cohort were fed with a high fat diet or with a normal chow for 16 weeks, starting at the age of 4 weeks. Second mice cohort were fed with a high fat-low methionine-choline deficient diet (A06071302 from Research Diets, Brunswick, NJ), used for NASH development, or with a normal fat control diet (A08051501 from Research Diets) for six weeks. Upon completion of the feeding period, a systemic perfusion of the mice with PBS was performed and livers were excised.

Chronic IL-6 treatments: Alzet osmotic pumps (Durect, Cupertino, CA) were used in mIndy L-KO and mINDy fl/fl littermate control mice. IL-6 was infused for 14 days with an infusion rate of 1  $\mu$ l/h (32  $\mu$ g/ml hIL-6 in 0.9% NaCl, 0.1% BSA). One week prior to the experiment, an intravenous line was inserted into the right jugular vein. On the day of the experiment, mice were fasted overnight and infused with 7 $\mu$ Ci  $^{14}$ C-Citrate continuously. After 4 hours, mice were sacrificed and livers were taken and snap frozen within 1 minute and stored at  $-80^{\circ}\text{C}$  for later analysis. Fatty acid synthesis from citrate was determined in these livers as described in (7).

**Hepatocyte preparation and cultivation**—Density gradient-purified hepatocytes were prepared without the use of collagenase, as described previously (12). Detailed information is given in the Supplemental Section.

**Microarray analysis**—Microarray data was analyzed using DIANE 6.0, a spreadsheet-based microarray analysis program based on JMP 7.0 from SAS system, as mentioned in our previous studies (7).

**Identification of transcription initiation sites by 5'-RACE**—Total RNA from two different primary human hepatocyte cultures was used with 5'-RACE system 2.0 (Life Technologies, Eggenstein, Germany) as described previously (12).

**Generation of human *mINDY* promoter constructs**—Fragments containing 618 bp or 376 bp of the putative human *mIndy* promoter were generated from human genomic DNA by PCR using specific primers. Fragments were cloned in the forward orientation into pGL3-basic (Promega, Mannheim, Germany). Luciferase-based reporter assays with transfected primary mouse hepatocytes were performed as described previously and compared against empty vector control transfected hepatocytes (12). Primer oligonucleotides and plasmids are given in the supplemental methods section.

## Statistical Analysis

Detailed information is given in the Supplemental Section.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

<b>Indy</b>	I'm Not Dead Yet
<b><i>mIndy</i></b>	mammalian homolog of Indy (gene, Slc13A5)

<b>mINDY</b>	mammalian homolog of Indy (protein, NaCT)
<b>IL-6</b>	Interleukin-6
<b>STAT-3</b>	signal transducer and activator of transcription 3
<b>ASO</b>	anti-sense oligonucleotides
<b>HFD</b>	high fat diet
<b>PI-3</b>	phosphoinositol 3
<b>MAPK</b>	mitogen-activated protein kinases
<b>NAFLD</b>	non-alcoholic fatty liver disease
<b>PCR</b>	polymerase chain reaction
<b>K<sub>m</sub></b>	Michaelis constant
<b>V<sub>max</sub></b>	maximum rate achieved by the system
<b>HOMA-IR</b>	homeostatic model assessment for insulin resistance
<b>SD</b>	standard diet
<b>HFS</b>	high fat high sucrose diet
<b>NASH</b>	non alcoholic steatohepatitis
<b>IL-6R<sup>L-KO</sup></b>	liver specific IL-6 receptor knockout
<b>LPS</b>	lipopolysaccharide
<b>ACC</b>	acetyl-CoA carboxylase
<b>NADPH</b>	nicotinamide adenine dinucleotide phosphate

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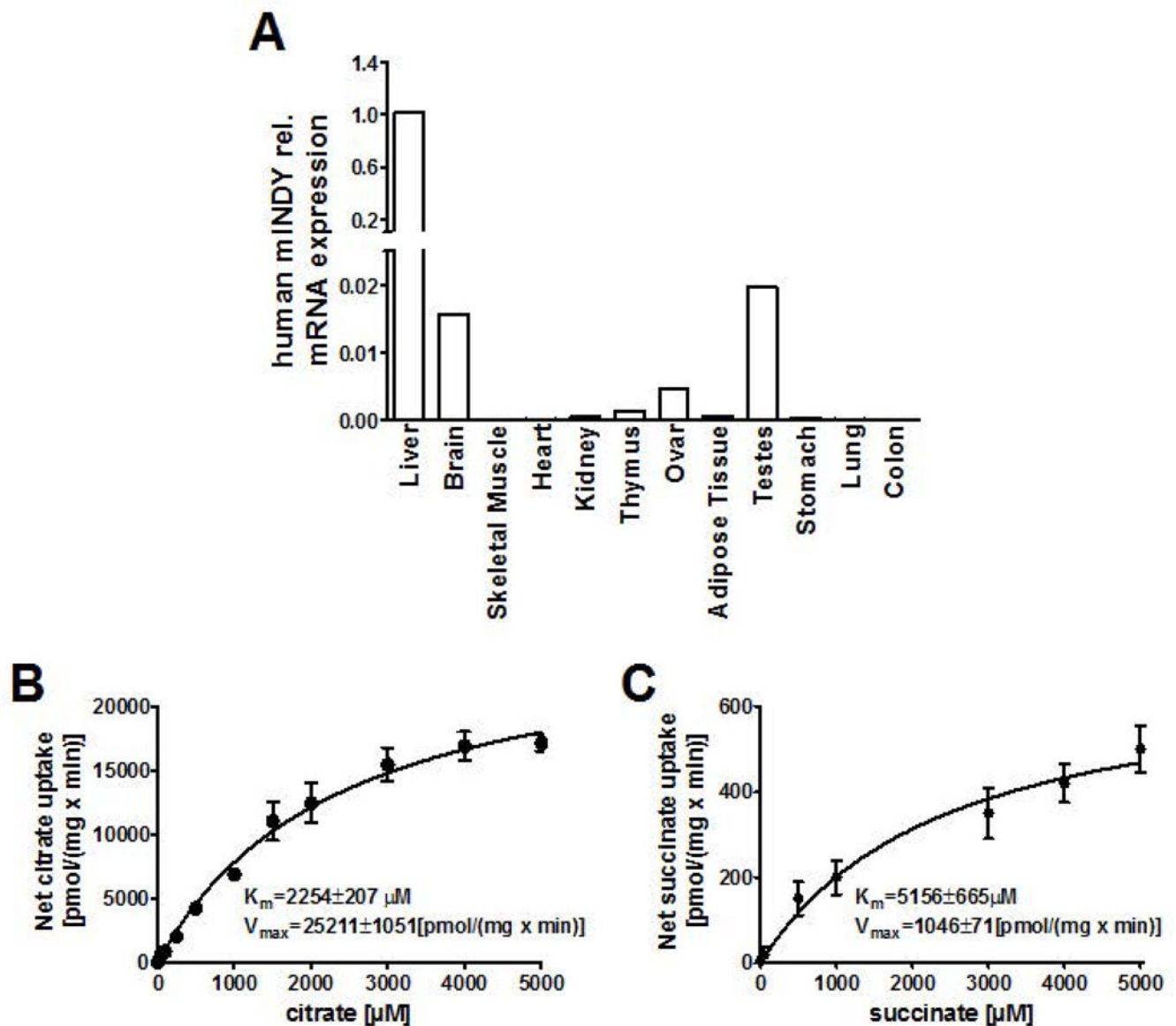
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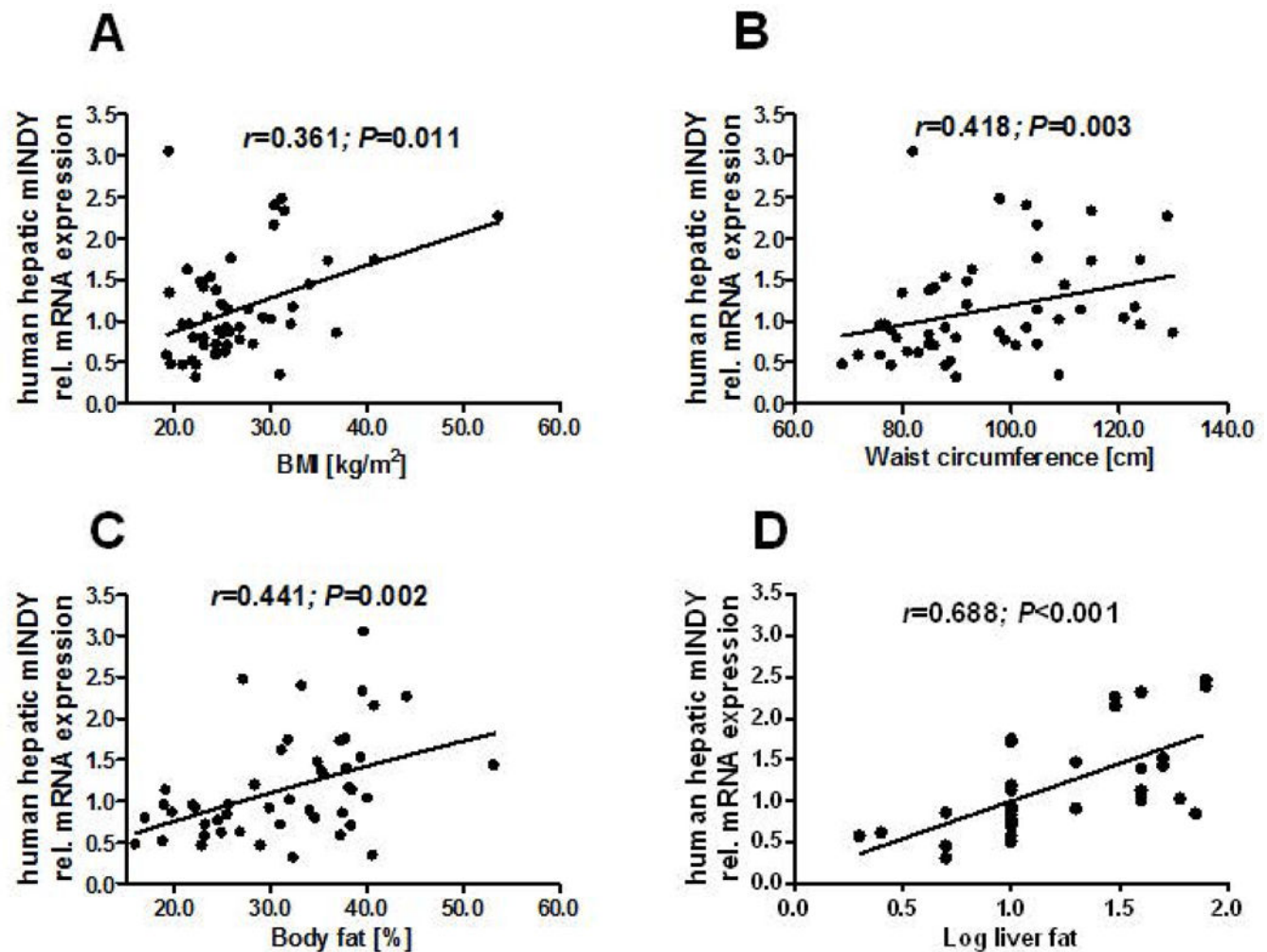
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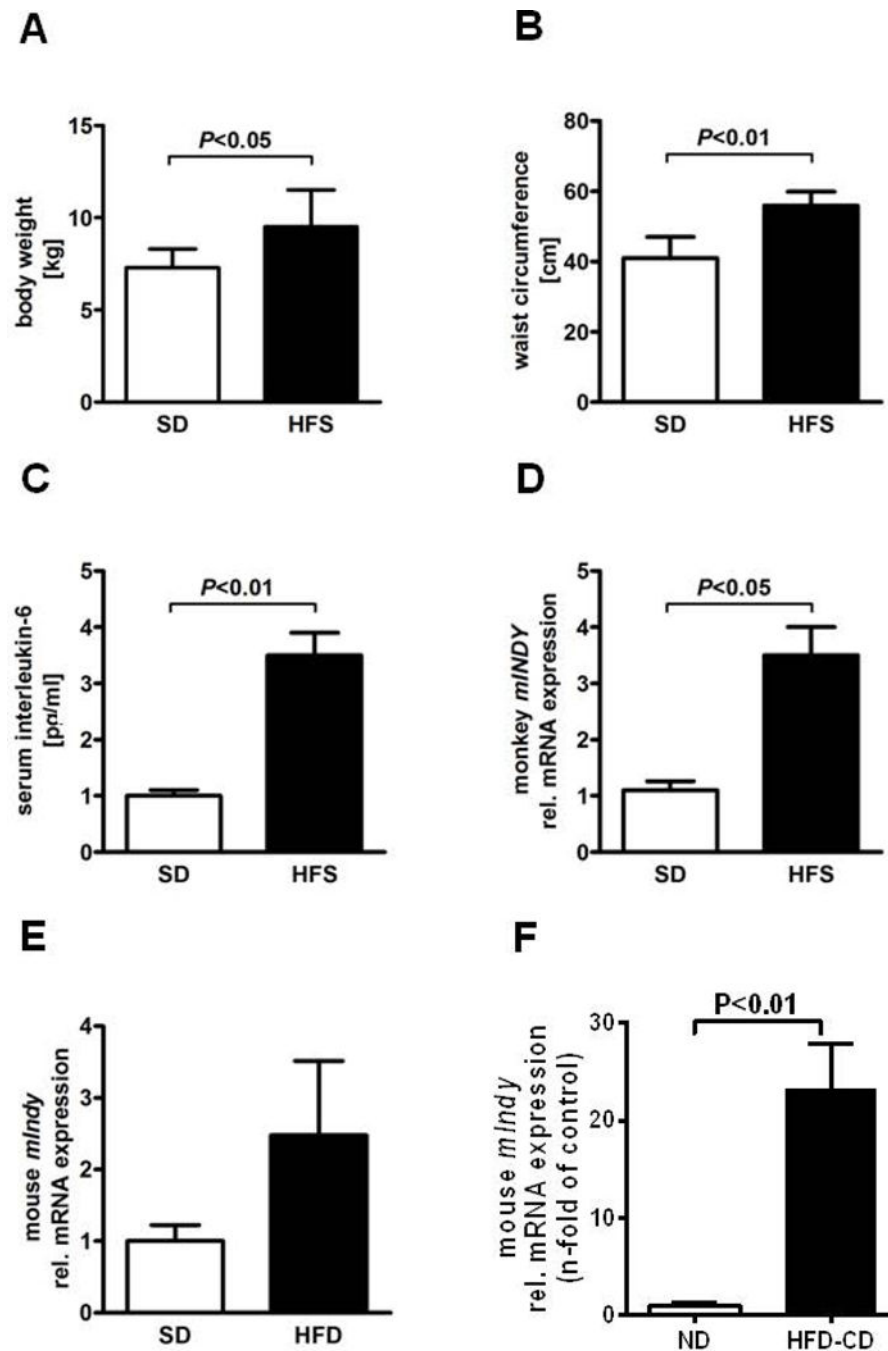


**Figure 1. Human *mIndy* tissue distribution and functional characteristics**

A) Human *mIndy* mRNA tissue expression in different tissues. B and C) *mINDY* transport kinetics for citrate (B) and succinate (C) were carried out in HEK293 cells transfected with either the *pIndy*-human.31 plasmid or empty expression vector pcDNA3.1(+). The net uptake is expressed as the difference between the uptake of substrates into HEK293 cells overexpressing human *mINDY* and pcDNA-transfected controls ( $n=3-6$  for each concentration).  $K_m$  values were determined by fitting the data to a non-linear regression curve fit.

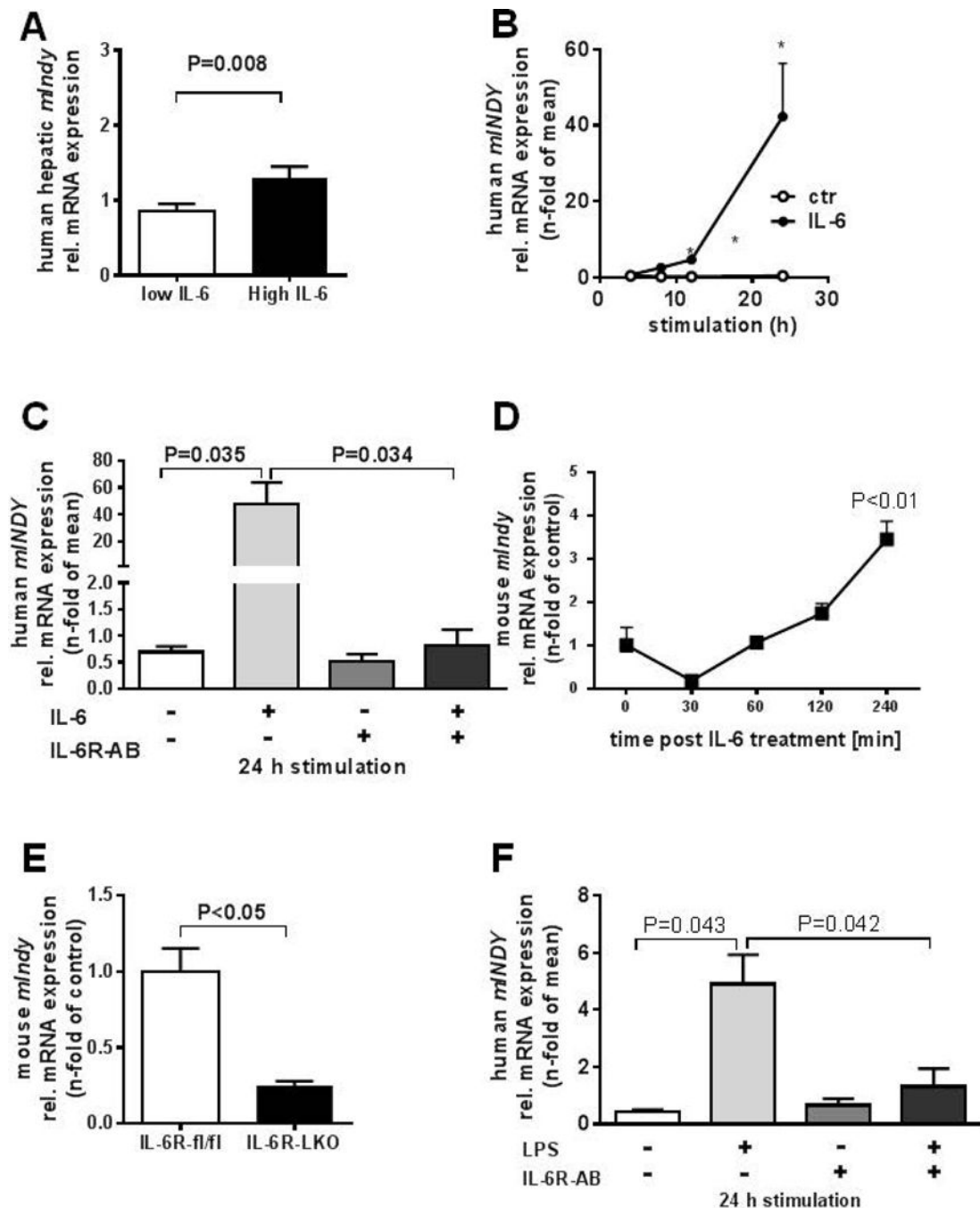


**Figure 2. *mIndy* expression in the liver of patients with different BMI and liver fat contents**  
 A) *mIndy* expression was positively associated with BMI, B) waist circumference, C) body fat, D) histologically assessed liver fat content. The degree of liver histology-determined steatosis remained significantly associated with *mIndy* expression after adjustment for several confounders (Model in Table 1), identifying *mIndy* as an independent risk factor for NAFLD in our cohort of patients.



**Figure 3. *mIndy* expression in liver tissue from non-human primates**

Rhesus monkeys were maintained on a standard diet (SD, n=4) or a high fat, high sucrose diet (HFS, n=10) for 24 months. Monkeys on HFS diet had A) increased BMI, B) larger waist circumference, C) elevated serum IL-6 levels, and D) increased hepatic *mIndy* expression compared to SD-fed animals. E) In non fasted male 16 week high fat diet fed C57BL/6 mice, hepatic *mIndy* expression was induced compared to the SD-fed group (n=4–5) F). In a murine NASH model (high fat/methionine low-choline deficient diet=HFD-CD), hepatic *mIndy* expression was markedly increased after 6 weeks of the intervention.



**Figure 4. *mIndy* interacts with IL-6**

A) IL-6 is a predictor of hepatic steatosis at cut-off level 4.81pg/ml (29, 30). Patients were divided into two groups with IL-6 levels below or above this threshold and *mIndy* expression in liver samples were determined. B) In human primary hepatocytes, IL-6 induced *mIndy* expression in a time-dependent manner (n=6). C) Blockade of the IL-6R with the monoclonal IL-6R antibody tocilizumab, completely abolished IL-6-mediated induction of *mIndy* (n=6). D) I.v. injection of IL-6 into C57BL/6 wildtype mice leads to an increase in *mIndy* mRNA levels (n=4). E) Targeted, liver-specific deletion of the IL-6R (IL-6R<sup>L-KO</sup>)



(n=3) in mice leads to a decrease in hepatic *mIndy* expression. F) Human non-parenchymal cells (including Kupffer cells) were co-cultivated with human primary hepatocytes and stimulated with lipopolysaccharide (LPS) with or without tocilizumab (n=3).

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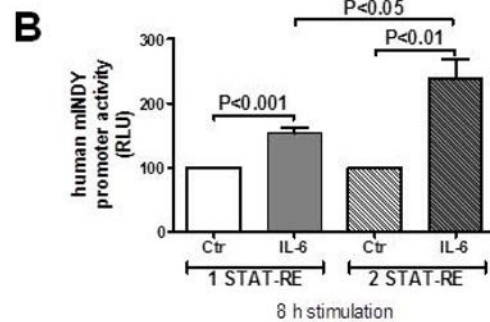
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**A** Human *mINDY* promoter

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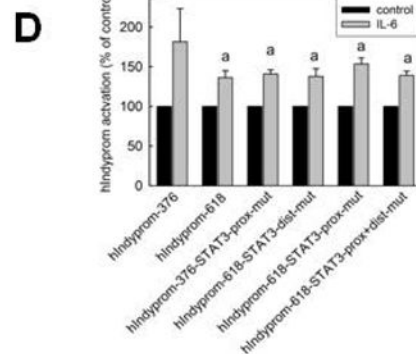
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GCCCCCAGTCTCCTCCCGCGCATGGCCTCGG

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**C** Mutation strategy

STAT3-consensus sequence : TTCTGGGAATT

**hindyprom-618-** cacctagagtggggcgagtgaggaagcc**STAT3dis**aggggggaccgggAA(tt)gaggagccgaggt  
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Agatagacctgagagaccagagggcctgagagagttcatccgggacccttcagagccaggaaagagcagatggcagcgggagggcg  
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CagcgaggagagggctggacagcgcgagatgctAggggccagctgccccctagagggctggccggaagcgttgagtccaaagagcctcc  
CaccggccggggggcagaattggggcgagcgcgtcCcacagaccccgaggggtggccccggcccgagggcgaggagggcgccccgt  
Gcggggcgaggtgtcaccgccccctccccaatccccgggagctgtggcccccttaagcccgggcgccctagctgccccctactgctcgc  
cgccaggtctcct

**Figure 5. Characterization of the human *mIndy* promoter**

The putative start site and upstream promoter region of the human *mIndy* gene were determined by 5' RACE (A) The most frequent transcription start site (bold) was located 40 bp upstream of the ATG start codon. (B) Reporter gene constructs were generated by cloning the 621 bp (prom-621, blue top) or 379 bp (prom-379, red top) sequence upstream of the ATG start codon of human *mIndy* in front of a luciferase reporter. C) Site directed mutagenesis strategy in 376 bp and 618 bp *mIndy* promoter fragments. D) Site directed

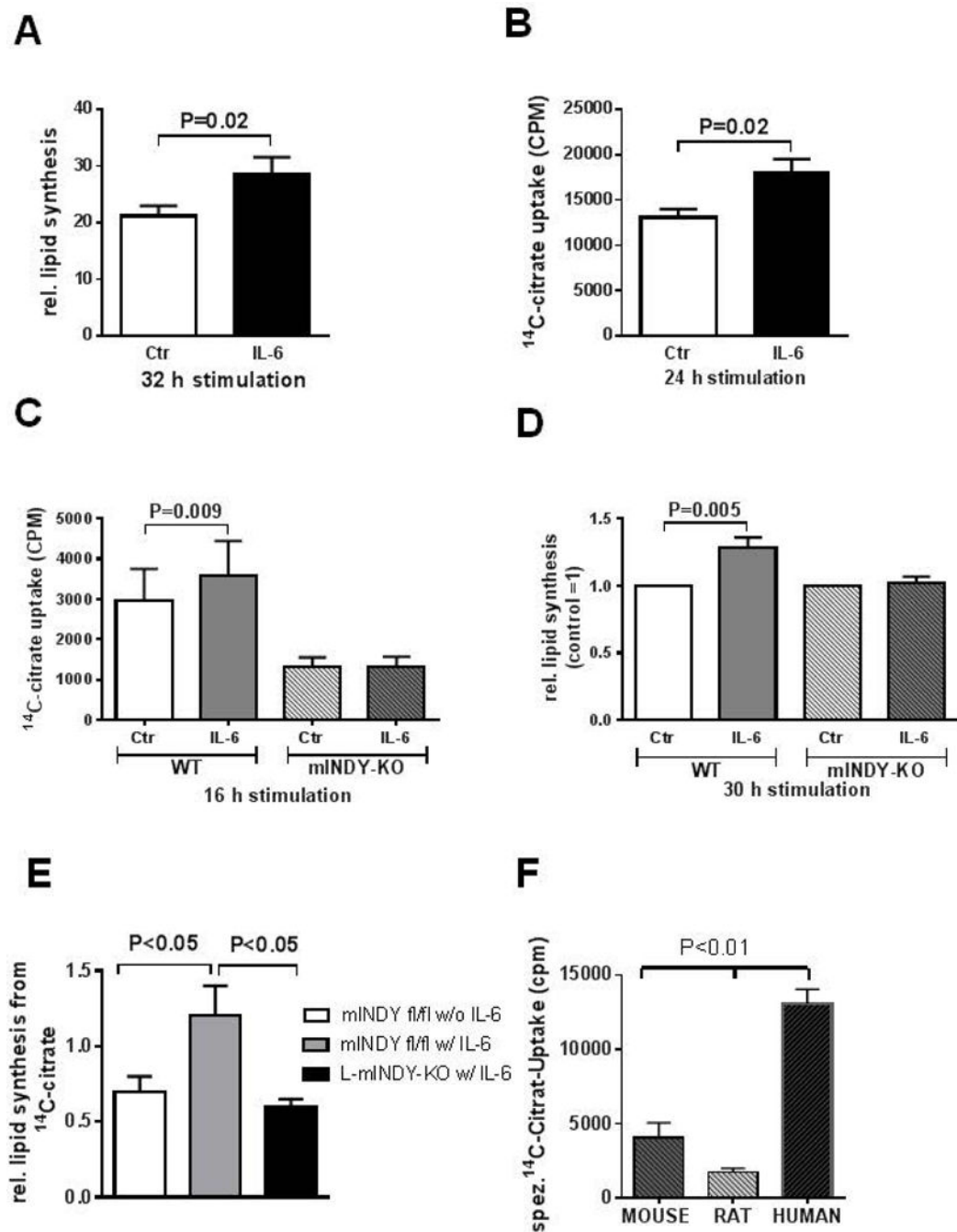
mutagenesis at STAT3-elements 376 bp and 618 bp in the mIndy promoter did not reduce promoter activity. Luciferase activity was determined and compared to empty vector control.

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**Figure 6. Cytoplasmic citrate uptake and lipid synthesis**

A,B) In human primary hepatocytes, IL-6 increased cytoplasmic influx of citrate (A) and lipid synthesis from citrate (B). C and D) IL-6 increased citrate uptake (C) and lipid synthesis from citrate (D) in a primary culture of hepatocytes from wildtype (WT) mice, but not from mice with deletion of *mIndy* (mINDY-KO). E) In liver specific mINDY KO mice (mINDY-KO) and mINDY fl/fl control mice, IL-6 was infused for 14 days via miniosmotic pumps.  $^{14}\text{C}$ -citrate was then administered iv for 4 hours and fatty acid synthesis was determined in liver of these mice. mINDY fl/fl mice without IL-6 infusion were used as

negative controls. IL-6 increased fatty acid synthesis from citrate, an effect prevented when mIndy was deleted in the liver. F)  $^{14}\text{C}$  Citrate uptake into primary mouse, rat and human hepatocytes incubated for 24h hours with citrate. Data represent values $\pm$  SEM from primary hepatocytes from independent assays from three different mice, 5 different rats and 5 different human donors.

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**Table 1****Human patient characteristics and model calculation**

n (male)	49 (23)
age (years)	59±2
BMI (kg/m <sup>2</sup> )	26.7±0.9
body fat (%)	32.9±1.4
waist circumference (cm)	96.1±2.3 <sup>a</sup>
SBP (mm Hg)	133±3
DBP (mm Hg)	73±2
AST/ALT	1.26±0.10
HOMA-IR	2.84±0.45
NAS (0–8)	1.72±0.24 <sup>a</sup>
Liver steatosis	16.66±3.22
steatosis score (0–3)	0.92±0.13 <sup>a</sup>
ethanol intake (drinks/week)	1.4±0.3

<b>Model</b>	
<b>Dependent variable: Hepatic steatosis</b>	
<b>Independent variable</b>	<b>Beta coefficient (P-value)</b>
	<b>R = 0.760; R<sup>2</sup> = 0.577, adj. R<sup>2</sup> = 0.496, P &lt; 0.001</b>
Sex	0.106 (0.45)
Age	0.123 (0.38)
Waist	0.279 (0.100)
Insulin Resistance (HOMA-IR)	0.285 (0.046)
Hepatic <i>mINDY</i> expression	0.442 (0.008)

Significant correlations are shown in bold.



**Table 2**Gene Ontology enrichment analysis in livers from subjects with high vs low *mINDY* expression.

Gene Ontology Term	Zscore	(P_value)	(fdr)
GO0042627 CHYLOMICRON	<b>10.084</b>	0.000	0.007
GO0042157 LIPOPROTEIN METABOLIC PROCESS	<b>9.829</b>	0.000	0.006
GO0046870 CADMIUM ION BINDING	<b>9.253</b>	3.18E-11	3.37E-09
GO0005319 LIPID TRANSPORTER ACTIVITY	<b>8.126</b>	0.002	0.034
GO0006953 ACUTE PHASE RESPONSE	<b>6.519</b>	0.003	0.042
GO0006572 TYROSINE CATABOLIC PROCESS	<b>5.016</b>	0.002	0.036
GO0004984 OLFACTORY RECEPTOR ACTIVITY	<b>4.036</b>	9.82E-19	2.38E-16
GO0005840 RIBOSOME	<b>3.848</b>	0.001	0.014
GO0003735 STRUCTURAL CONSTITUENT OF RIBOSOME	<b>3.684</b>	0.001	0.021
GO0008270 ZINC ION BINDING	<b>-8.407</b>	3.40E-13	4.44E-11
GO0003677 DNA BINDING	<b>-8.592</b>	9.26E-13	1.12E-10
GO0006350 TRANSCRIPTION	<b>-9.520</b>	4.12E-15	6.35E-13
GO0046872 METAL ION BINDING	<b>-9.790</b>	8.75E-16	1.41E-13
GO0016020 MEMBRANE	<b>-9.979</b>	4.72E-17	8.89E-15
GO0006355 REGULATION OF TRANSCRIPTION DNA DEPENDE	<b>-10.015</b>	7.29E-17	1.30E-14
GO0005737 CYTOPLASM	<b>-11.918</b>	6.03E-21	1.86E-18
GO0005515 PROTEIN BINDING	<b>-14.315</b>	4.65E-29	1.97E-26
GO0005634 NUCLEUS	<b>-14.608</b>	1.50E-33	1.02E-30

Gene Ontology terms of biological processes. Positive z-score indicates increased in patients with high *mIndy* expression. Negative z-score indicates reduced in patients with high *mIndy* expression.